STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

TWENTY-FIVE YEARS OF COÖPERATION WITH THE U. S. DEPARTMENT OF AGRICULTURE

As 1946 has witnessed the termination of formal coöperation between the Stain Commission and the U. S. Department of Agriculture, this seems a good time to review the relationship which has existed between the two bodies for a full quarter century. This coöperation started even before the Stain Commission was formally organized, and has continued ever since. Even now that it has officially come to an end, it is expected that coöperation with individual members of some of the Department Laboratories will continue.

BEGINNINGS OF STAIN INVESTIGATIONS IN THE UNITED STATES

In 1920 the inadequacy of biological stains on the American market was beginning to be realized, and at least two independent attempts to do something about the subject were undertaken. The first of these was a letter written by R. T. Will of the Will Corporation of Rochester, New York to the Color Laboratory of the U. S. Bureau of Chemistry at Washington, asking whether they had "undertaken any work with the thought of assisting scientific investigators in pathological, biochemical and bacteriological fields in the preparation or the purification of dyes of American manufacture for scientific purposes"; also, whether they were "able to undertake, either the manufacture of new dyes or purification of those available". This letter was very courteously answered by Dr. C. O. Johns, then Chemist in Charge of the Color Investigation Laboratory, stating his interest, but also expressing his fear that nothing could then be accomplished along such lines, owing to a decreased appropriation and the resulting cut in personnel.

While this correspondence was going on, an entirely independent set of inquiries was being made on the part of the Society of American Bacteriologists as to whether there might not be some way of investigating and improving American stains. As a result, Dr. A. P. Hitchens, then Secretary-Treasurer of that Society, approached the writer, as chairman of the Committee on Bacteriological Technic, to see

whether it might not be a matter within the scope of that committee. The writer agreed that it was, but mentioned practical difficulties in the way of organizing such an investigation.

A little later that year (October), by a lucky chance, these two lines of attack were brought together. The writer began having trouble with a certain dye (rose Bengal) of American manufacture, and the letter of inquiry on the subject, addressed to the Will Corporation, came to the attention of R. T. Will. The latter promptly expressed his interest in seeing some sort of standardization of American stains; and was informed that this very matter had been referred to the Committee on Technic. Mr. Will, therefore, decided that this Committee offered more immediate opportunities of accomplishing his objects than did the Department of Agriculture. A coöperative project to investigate certain American stains was organized by the Committee, with the coöperation of the Will Corporation to secure the necessary commercial samples to investigate.

Mr. Will, moreover, had not been discouraged by his earlier unsuccessful efforts to secure the coöperation of the Department of Agriculture and began pursuing other lines of attack in trying to get the investigation put on a broader basis. This time, his line of approach was through the National Research Council, and it resulted more successfully.

ORGANIZATION OF THE WORK UNDER THE NATIONAL RESEARCH COUNCIL

During 1921, considerable correspondence was carried on relative to organizing work of this sort under the National Research Council. It proved that Dr. McClung, the retiring chairman of the Division of Biology and Agriculture, was much interested and had been trying himself to develop a method of producing hematoxylin more satisfactory than the American product of that day. The project was therefore referred to his successor as chairman of that Division, Dr. L. R. Jones. The Research Council had better facilities for organizing coöperative work of this nature than had any body previously approached. Most important for assuring its success was the fact that active coöperation of the Chemical Foundation and of the Department of Agriculture was secured.

By this time, the Color Investigations Laboratory was in charge of Dr. J. A. Ambler, who was invited to be present at the first conference called on the subject. This conference was held on November 5, 1921. The result of the conference was the appointment of a Committee on Standardization of Biological Stains, which operated for about one year under the Research Council and succeeded in get-

ting the work organized. This was done in very close coöperation with the Color Laboratory under Dr. Ambler. His Laboratory made a large number of chemical and spectrophotometric analyses of stains, both satisfactory and unsatisfactory, and the Committee endeavored to find some correlation between such findings and their performance in actual use.

ORGANIZATION OF THE STAIN COMMISSION

If it had proved possible, as at first hoped, to find some correlation between performance and chemical or optical properties, standardization might have been accomplished by drawing up chemical and physical specifications. The early investigations soon proved, however, that such a course was out of the question, at least for the time being; accordingly, the only practical course seemed to be to issue some form of certification to those manufacturers whose products proved satisfactory. Such a plan seemed eminently desirable to all concerned; but it clearly was not one of the functions of the Research Council to act as a certifying body for commercial products. It was accordingly decided that some independent body should be set up to serve such a purpose. Hence, the Commission was formed as an independent organization affiliated with the Research Council; the full name given to it was then the Commission on Standardization of Biological Stains.

In order to give this organization more weight of authority than that derived from the individuals composing it, the plan was adopted of having its Executive Committee composed of representatives of various national scientific societies. Dr. Ambler became a member of this committee, when it was first organized, as a representative of the American Chemical Society. Serving in this capacity, he was also unofficially a representative of the U. S. Department of Agriculture, and took charge of the coöperative work carried on at the Color Investigations Laboratory. At first, this coöperation was wholly informal, but it was later covered by a formal Memorandum of Understanding, signed by representatives both of the Department and of the Commission. This agreement has been renewed every year up to 1945, and has thus continued through several changes in the internal organization of the Department; hence, although there has been a continuity in personnel representing the Department, the agreement has formally been drawn up with first one subdivision and then another of the Department. Whatever the name of the subdivision, however, the coöperation has always been given heartily on the part of the Department, even though all the other of the original color investigations came to an end long ago, and of recent

years the logic of continuing the stain investigations as part of the Department's program has been open to question.

EARLY PHASES OF THE COOPERATION

When the work was first organized, the Commission had no financial resources, and the funds appropriated to it by the Chemical Foundation were merely enough to cover a small amount of clerical work and travel expenses. Under such circumstances, the Department of Agriculture offered not only the facilities of the Color Laboratory, but the services of the assistants necessary to do the chemical work. In a short time, in fact, they secured a well-trained young dye chemist, W. C. Holmes, who gave the greater part of his time, as an employee of the Department, to the studies of biological stains; and later he was assisted in this work by one or two others, notably, J. T. Scanlan.

The early phases of the work consisted largely of developing methods for analysis of stains, and the routine assay of samples submitted by the companies; it was obvious that the former undertaking had to precede the latter. Few methods were known at the beginning of the work, so they had to be developed for one or two dyes at a time, and no stain could be put on the certification basis until the preliminary work on methods had been done. As a result, the Color Laboratory was doing the lion's share of the entire work on stains during the first stages of the coöperation.

It should also be mentioned that the first Edition of Biological Stains was published in 1925. Dr. Ambler gave much assistance in writing that book, and can be regarded as most outstanding among the collaborators in that undertaking.

In the late twenties changes began to occur. Dr. Ambler left the work, and the laboratory of which he had been in charge became primarily devoted to the utilization of farm wastes, the color investigations becoming a minor part of its activities. For several years, however, the coöperation of the laboratory with the Stain Commission remained as active as ever; when Dr. Ambler left, the biological stain investigations were put in charge of Mr. Holmes, who became the American Chemical Society's representative on the Commission. In this capacity he became one of the Commission's most active members, and gave very valuable assistance not only in the analytical work but also in the revision of the book, Biological Stains. The second edition of this book appeared in 1929, and it was thanks more to him than to anyone else that it became a very much more useful contribution to the field than had the first edition; he gave invaluable services in ironing out errors that had occurred in the first edition,

largely because it had been written by individuals having so little personal knowledge of dye chemistry.

In 1930 the Commission for the first time employed a chemist of its own; this was Miss A. R. Peterson—now Mrs. Bradshaw. She was first hired to investigate certain special problems in which the Chemical Foundation was sufficiently interested so that they were glad to finance the additional expense; but after completing those investigations, she stayed on as the Commission's analyst. She did all her work in the laboratory which had been carrying on the Department's color investigations. At first, she was under Mr. Holmes' direct supervision, and in association with Dr. Scanlan. Mr. Holmes' sudden and untimely death in 1932 and a protracted abscence of Dr. Scanlan, due to poor health, resulted for a year or so in putting the entire burden of the chemical work on Mrs. Bradshaw's shoulders. Later, as a result of the experience thus gained, she was able to take over the entire chemical work, although Dr. Scanlan's advice in regard to puzzling points has been in constant requisition ever since the time he recovered his health. During this period the representative of the American Chemical Society on the Commission was first Dr. H. T. Herrick who, as Chief Chemist in Charge of the Color and Farm Waste Division, was Dr. Scanlan's immediate superior; while later Dr. Scanlan himself became the representative and has served as such until the present year.

When later the Department was further reorganized and Dr. Scanlan's time was needed wholly in connection with their problems on the utilization of farm products, Mrs. Bradshaw assumed even greater responsibilty in connection with this work. From that time on, the coöperation of the Department consisted chiefly in furnishing the Commission laboratory space and equipment; but although its contributions to the work did not any longer include the time of members of its staff (except in an advisory capacity), the coöperation still remained extremely valuable.

MOST RECENT PHASES OF THE COOPERATION

In 1940, the entire staff of what had once been the Color Laboratory was moved to Philadelphia as part of the staff of the Eastern Regional Research Laboratory, and the question came up as to what should then be done with the biological stain investigations, which hardly fitted into the program of that Laboratory. After much discussion, however, it was decided that the work was important enough so that the coöperation should be continued at least for the time being. As a result, the biological stain work was furnished laboratory space in the new building, and has been located

there for four or five years—after an intervening period of about a year, pending the completion of the Eastern Regional Laboratory, when the work was being carried on at the Philadelphia College of Pharmacy and Science. The coöperation has still been active during this period; and the location at Philadelphia had the advantage to the Commission of offering the advisory services of Dr. Scanlan who was a member of the Regional Laboratory staff.

Now the coöperation has come to an end, but with the best of feeling on both sides. The resignation of Mrs. Bradshaw last year has forced certain changes; and it seemed well to take the occasion to initiate other changes. The result eventually is going to be more complete centering of all the Commission activities in one place. It is with regret that the long association with the Department has been brought to an end; but making the change seems at present the wisest course.

This occasion is taken to express publicly the appreciation of the Commission to the Department of Agriculture for a quarter century of active and invaluable coöperation.—H. J. Conn.

PRECOOLING COMBINED WITH CHROM–OSMO–ACETIC FIXATION IN STUDIES OF SOMATIC CHROMOSOMES IN PLANTS

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ABSTRACT.—**The treatment of living excised roots in vials partially filled with water at 0°C. for 1½ hours before fixation in cold Benda fluid was found to shorten chromosomes and to preserve details of chromosome morphology in a number of plant species.**

The recent announcement in this journal, by Hill and Myers,[2] of a technic in which cold treatment is used to contract somatic chromosomes, prompts the description of a somewhat similar technic that has been in use for some time in the author's laboratory. Roots are taken from young greenhouse-grown plants shortly after the tips emerge from the ball of soil within the pots. Straighter and more rapidly growing roots may be obtained if the plants are thoroughly watered and the pots removed and replaced a day prior to fixation. The removal of the pot provides an air space between the pot and soil into which the young roots rapidly grow. The required number of roots is removed from a plant by snipping off the exposed tips ½ to ¾ inch in length with a fine forceps. These are placed in a Syracuse dish containing water, and any adhering soil removed by means of small camel's hair brushes. The clean roots are then transferred, along with an identification number, to an open 12×50 mm. shell vial partially filled with distilled or pure spring water. The vial is immediately cooled by placing it in an ice bath at 0°C. For this purpose a glass battery jar containing ice cubes and water and placed in an ordinary refrigerator has been found convenient. The vials with water and living roots are partly immersed and held in an upright position in the bath in a rectangular board provided with a number of depression holes.

After a given period of precooling—an hour and a half has been found optimum for most materials—the water is poured off the roots and replaced with Benda fluid, also cooled to 0°C. The vials are

[1]On leave from Department of Genetics, Carnegie Institution, Cold Spring Harbor, N. Y.

[2]Hill, Helen D., and Myers, W. M. A schedule including cold treatment to facilitate somatic chromosome counts in certain forage grasses. Stain Techn., 20, 89–92. 1945.

then stoppered and allowed to remain in the bath for an additional 2–3 hours, after which time they are removed to room temperature where fixation continues overnight. The Benda formula used contains 1% chromic acid, 16 ml.; 2% osmic acid, 4 ml.; and glacial acetic acid, 2 drops.

Dehydration, staining, and the application of pressure to the cover glass after mounting in balsam, to cause flattening and separation of the chromosomes, are the same as in a previously published schedule[3]. The crooked proximal portions of roots may be trimmed off with the needles at the time of embedding, and the meristematic tips aligned in parallel groups of 5 or 6 for cross-sectioning as a unit. The author generally prefers to place the roots of a single plant on a slide and does not glue them to cards, but this could be done if desired.

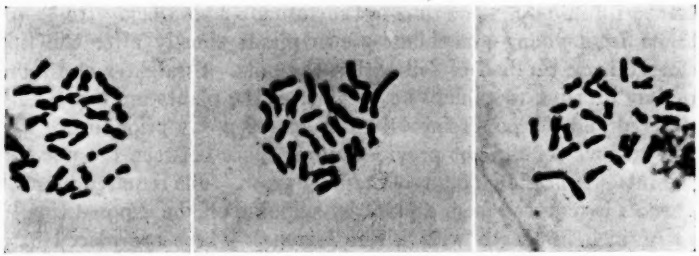

FIG. 1. Photomicrographs of somatic chromosomes from cold-treated roots of *Melandrium*, × 1400.

The effect of low temperature in shortening somatic chromosomes was discovered accidentally during attempts to induce "nucleic acid starvation" by cooling whole plants, after the method of Darlington and La Cour[4]. Precooling the living roots in small vials makes possible the treatment of roots from large numbers of plants without extensive refrigeration equipment. It also permits a short treatment time to be used; since the roots rapidly attain the desired low temperature. The latter is an important consideration because the number of cells in division is not appreciably reduced by the treatment; chromosome shortening apparently is induced in cells already in the process of division. The fact that the roots are removed from the

[3]Warmke, H. E. A section-smear method for plant cytology. Stain Techn., 16, 9–12. 1941.

[4]Darlington, C. D., and La Cour, L. Nucleic acid starvation of chromosomes in Trillium. J. Genetics, 40, 185–213. 1940.

plants before the cold treatment was found not to affect adversely the number of divisions or the fixation image.

Benda fluid is used because of its superior preservation of details of chromosome morphology, especially the centromere constrictions. The tendency of the chrom-osmo-acetic mixtures to give elongated and often overlapping somatic chromosomes, however, is completely overcome by the cold treatment. The combination of a fixing fluid that preserves details of chromosome structure, along with the shortening caused by cold, has proved tremendously useful in cytological studies of somatic chromosomes in this laboratory.

This technic has been used with excellent results on many different grasses, including species and varieties of *Panicum*, *Paspalum*, *Stenotaphrum*, *Penisetum*, and *Cymbopogon*, as well as on *Melandrium* and *Saccharum*. In *Melandrium* (Fig. 1) the centric constrictions are sufficiently well preserved so that it has been possible to make a study of spontaneous fragmentation of the Y chromosome and to correlate broken chromosomes with abnormalities in male development. These studies have shown that both arms of the Y may be involved, either singly or together, and that deficiencies may vary in length from small losses through a series of types to what appears to be the loss of a complete or nearly complete arm.

A MODIFIED FEULGEN TECHNIC FOR SMALL AND DIFFUSE CHROMATIN ELEMENTS

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ABSTRACT.—**A revised technic is proposed which differs essentially from the standard procedure only in the preparation of the staining solution and of the sulphurous acid bath, which are made by direct charging with SO_2 gas instead of HCl and the sulphites ordinarily used. Tests on smear preparations of small amoebae, oöcyte prophases of a parasitic wasp, and yeasts revealed Feulgen-positive results not usually obtained by the conventional method.**

INTRODUCTION

In an attempt to study the behavior of chromatin in the mitotic sequence of small amoebae, the Feulgen technic given in McClung (1937) was employed; other methods (e.g. deTomasi's) were also investigated. However, except for the compact chromosome stages at metaphase, negative results usually were obtained. It was thought, therefore, that without altering the principles of the Feulgen reaction, some unessential and perhaps detrimental components might be removed from the usual reagents used and so a better reaction in the small, more diffuse chromatin elements difficult to stain would be promoted. Hence, a method was adopted which differs essentially from the standard technic only in the manner of preparing the staining solution and the rinsing bath. A decided improvement in delicacy of selectivity was immediately realized.

METHOD

Hydrochloric acid apparently is necessary only to release sulphur dioxide from the sulphites ordinarily used in the formation of leuco basic fuchsin and in the sulphurous acid bath following staining. Both the acid and the sulphite were therefore eliminated, and direct charging of both the basic fuchsin and the bath water with sulphur dioxide gas was substituted. Normal hydrochloric acid was employed only in the necessary process of hydrolysis.

By gentle bubbling of sulphur dioxide gas from a small aperture in glass tubing into 100 ml. of 0.5% basic fuchsin solution, decolorization takes place in about an hour and the reagent is then ready for

use. Distilled water is similarly saturated for the sulphurous acid rinsing bath and may be stored for weeks in a tightly-corked flask. The sulphur dioxide gas can be produced easily by a simple flask-and-funnel generator using sodium bisulphite and dilute sulphuric acid.

The reagents thus made were then used according to the slightly modified standard procedure as follows:

1. Fixation, mostly 2 to 20 minutes. (Time is for smear preparations).
2. Washing, minimum time according to the fixative used (not over 20 minutes).
3. Distilled water, 2 minutes.
4. Normal HCl at room temperature, 2 minutes.
5. Normal HCl at 60°C., 8 to 10 minutes.
6. Normal HCl at room temperature, rinse.
7. Distilled water, rinse.
8. Sulphurous acid, 2 minutes.
9. Leuco basic fuchsin, 1½ to 2 hours.
10. Sulphurous acid bath, for sufficient time to remove the free unreacted leuco basic fuchsin (usually two or three 1-minute changes).
11. Tap water, 10 to 15 minutes.

Counterstaining with fast green may be in either aqueous or alcoholic solutions. Dehydration is accomplished either through the alcohols or from water through triethyl phosphate (Nelsen, 1945) directly into xylene. The latter is a shorter method and does not appreciably remove the aqueous counterstain.

Discussion

The foregoing method was tested on smear preparations of three types of organisms: (1) various small, endosome-containing amoebae; (2) germinal cells of female parasitic wasps, *Habrobracon;* and (3) the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. The fixatives used were: for amoebae, either Schaudinn's or weak Flemming's; for *Habrobracon*, formalin-acetic-alcohol; and for yeast, chrome-acetic-formalin. Fixation time was restricted to as low as two minutes and to not more than twenty minutes, with the exception of *Habrobracon* which was kept in the fixative for from one to about three hours. A minimum time of fixation and washing in water as well as a minimum preservation time in the alcohols was found most favorable. Also, a basic fuchsin which becomes decolor-

ized to a light straw or even a lighter hue proved to be best; see deTomasi (1936)[1].

The different species of amoebae registered all the mitotic phases including very delicate and diffuse chromatin and so presented a complete picture of the mitotic series.

For *Habrobracon*, the staining method was used both by Dr. Anna R. Whiting of this laboratory and by the author. During the growth period, the oöcyte nucleus (prophase I) representing diffuse chromosomes did not stain by the conventional procedure (Speicher, 1936, and A. R. Whiting, personal communication), but did show up distinctly when the above described method was tried.

The yeasts, grown on Sabouraud's agar, clearly showed in the vegetative stages not only a densely-stained, Feulgen-positive "centriole" or "nucleolus", but also Feulgen-positive chromatin in the form of threads and granules in the "nuclear vacuole".

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DeTomasi, J. A. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

McClung, C. E. 1937. Handbook of Microscopical Technique. Paul B. Hoeber, Inc., New York.

Nelsen, O. E. 1945. The use of dimethoxytetraethyleneglycol and triethyl phosphate in histological technic. Stain Techn., **20**, 131–2.

Speicher, B. R. 1936. Oögenesis, fertilization and early cleavage in *Habrobracon*. J. Morph., **59**, 401–21.

[1]The lots of dye used most constantly in this work were a Grübler sample and one (Cert. No. CF–17) from Coleman and Bell. Four other batches, bearing certification numbers CF–5, CF–8, CF–27, and LF–7, were also employed. Three batches, NF–49, NF–52 and NF–53, which had been approved by the Stain Commission only for purposes other than the Feulgen stain, proved usable, but the color of the Feulgen-positive bodies was somewhat brownish, and the reaction was not sufficiently intense.

AN OXIDATION AND ADSORPTION REACTION FOR DIFFERENTIATING THE ENDODERMIS AND THE COLLENCHYMA

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Abstract.—**A method is presented for a study of the endodermis and collenchyma in vascular plants. The method is useful in histologically isolating these tissues, particularly where the accepted anatomical features and the usual staining reactions are not definitive. The exclusive oxidation of the leuco (reduced) form of common redox indicators to the oxidized and colored form by the endodermis may be induced by treating fresh free-hand or freezing microtome sections with various alkaline salts at pH 8.5 to 9.0. Placing sections in sodium borate or sodium selenite induces an immediate oxidation of a tincture of gum guaiac in the endodermis and after several minutes a gradual and weak oxidation in the collenchyma. Addition of glycerol to sections which show oxidation in the endodermis induces a localized adsorption of the oxidized indicator in the collenchyma. The endodermis and the collenchyma may be differentiated best by oxidation and adsorption respectively by means of sodium selenite, guaiac and glycerol in the order given. The collenchyma in roots has not been defined to date, and the reactions described introduce a new method of study for this tissue. By treating sections with boric acid a gradient in the guaiac reaction appears in the cortex.**

The unsaturated fat oxidation system of the endodermis has been described,[1] and the work presented here is an application of redox studies in isolating oxidases[2] to the problem of isolating the tissues noteworthy for their high content of unsaturated fat oxidase. Any oxidase indicator (guaiac, paraphenylenediamine, etc.) or any reduced (leuco) redox indicator will be oxidized initially and exclusively by the endodermis if sections are first treated with sodium selenite or sodium borate. Alkaline salts activate the oxidation of indicators by the endodermis, but other oxidation systems are depressed so that the localized oxidation reaction is a simple method of histologically isolating the endodermis.

[1]Van Fleet, D. S. 1942. The significance of oxidation in the endodermis. Amer. J. Bot., **29**, 747–55.

[2]Van Fleet, D. S. 1943. Unsaturated fat oxidase: distribution, function and histochemical identification in plant tissues. J. Amer. Chem. Soc., **65**, 740.

PLATE 1

Explanation of Figures

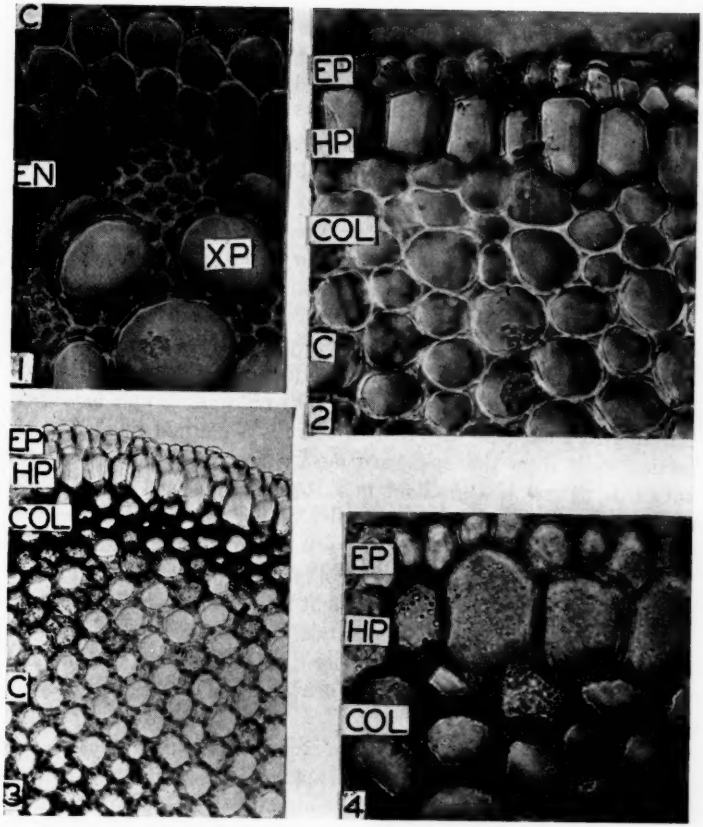
Photomicrographs of root sections (100μ thick) of *Allium Cepa var. solanicum*.

FIG. 1. The dark shaded zone in the endodermis (EN) is the localized oxidation of gum guaiac following a one minute treatment with sodium borate. The xylem polar groups (XP) and alternate patches of phloem inside the stele do not react with guaiac until after 8–10 minutes, and the cortical cells (C) outside the endodermis do not react until 5–10 minutes after the initial reaction in the endodermis as shown in this photograph. × 218.

FIG. 2. Epidermis (EP), hypodermis (HP), collenchyma (COL) and underlying cortical cells (C) unstained prior to reaction with gum guaiac. The characteristic histological features of the collenchyma (COL) with walls thickened in the corners may be seen as compared with the underlying typical cortical parenchyma cells (C). × 295.

FIG. 3. The localized adsorption of gum guaiac in glycerol by the collenchyma (COL) is confined to this tissue and does not occur in the overlying dermal layers (EP and HP) or the cortical parenchyma (C). × 190.

FIG. 4. An enlargement of the section shown in figure 3 to indicate the adsorption of oxidized guaiac in glycerol on collenchymal walls (COL). × 300.


C
EN
XP
1
EP
HP
COL
C
2
EP
HP
COL
C
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COL
4

A 5% tincture of gum guaiac in 95% ethyl alcohol is boiled in Norit-A for 10 minutes to remove peroxides in the guaiaconic acid. A drop of gum guaiac tincture is applied to a fresh free-hand or freezing-microtome section and there is a generalized blueing (oxidation) of the guaiaconic acid by all of the cells in the section, but if the sections are treated for one minute with a 2% solution of sodium borate, sodium selenite, or sodium dibasic phosphate before applying the guaiac the reaction is confined to the endodermis for 5 to 10 minutes depending on the plant used. NaOH/HCl buffers at pH 8.5–9.0 will also induce a localized guaiac reaction confined to the endodermis (Fig. 1).

After standing in guaiac for 5 to 10 minutes there is a slow and gradual reaction in the collenchyma (Figs. 2, 3 and 4) that may be intensified by adding a drop of glycerol to the section and an additional drop or two of guaiac tincture. The addition of glycerol brings about the localized adsorption of oxidized guaiac (tetraguaiaconate) on the walls of collenchymal cells in root sections (Figs. 3 and 4). A differentiating stain for root collenchyma has not been described to date and the adsorption of oxidized guaiac by collenchymal cells introduces a new method of study for this tissue.

After sections have been treated with mild alkali and have been standing in guaiac for 10 minutes there is a gradual blueing of the cortical cells from the epidermis inward and from the endodermis outward. There is a gradient in this guaiac reaction in the cortex from the epidermis toward the center of the cortex. This gradient is best demonstrated by treating sections with boric acid (pH 5.6) followed by guaiac. After the initial reaction in the endodermis and collenchyma there is a cortical gradient reaction as follows: epidermis 1, 2, 3, 4, 5 — 5, 4, 3, 2, 1 endodermis. There are two waves of reaction, one from the epidermis inward, the other from the endodermis outward, and both meet in the center of the cortex, and they are mirror images.

In addition to the use of gum guaiac more careful studies may be made with common redox indicators applied in the colorless (reduced) form. Methylene blue, indigo carmine, 2–6 dichlorophenol-indophenol or brilliant cresyl blue may be reduced by means of sodium thiosulfate or sodium metabisulfite or by hydrogen gas bubbled through platinized asbestos. These indicators are more sensitive to free oxygen, and they must be applied under nitrogen in a hanging drop gas chamber or in a modified van Tieghem cell.

A RAPID FREEHAND SECTIONING METHOD FOR LEAVES[1]

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ABSTRACT.—**For the rapid sectioning of such material as fungus leaf spots a method has been evolved whereby a piece of leaf is soaked in lactophenol and sections are sliced off it, on the slide, under the dissecting microscope, by means of a diagonal scalpel ground with a slightly curved blade. Poor sections can be recognized and removed as soon as they are cut; and it is commonly possible at the same stage to distinguish which sections contain fruiting elements of a fungus.**

During studies of the leaf smuts belonging to the genus *Entyloma*, and of other leaf parasites that require sectioning for adequate study, the writer has found that the preparation of a mount by sectioning in pith frequently takes longer than the examination of the mount and seriously limits the number of specimens that can be examined in a day. In addition, the method keeps the operator in some doubt as to the quality of the sections until the procedure has been completed, and the thinnest sections tend to be damaged by the requisite handling.

The method about to be described has been used by the writer for the examination of several hundred specimens during the last two years. It allows the complete preparation of a mount with a number of oriented sections, from selection of a lesion to application of the cover glass, in fifteen minutes or less. It also allows generous mounts to be made from a minimum of material, often an important consideration in the study of scanty and valuable specimens.

Presumably many workers have at one time or another tried to speed up section-cutting by some means, and some have probably used the dissecting microscope for the purpose. No doubt certain phases of the writer's method have been developed independently by others; but no description has been found in print of a similar process; and inquiry at several institutions has led to the belief that various features are sufficiently novel, and the advantages sufficiently great, to make publication advisable.

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[1]Contribution No. 848 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2]Junior Plant Pathologist.

A promising lesion is selected under the dissecting microscope, the area is soaked successively with 95% alcohol and water if dried material is being examined, and a piece is cut out. The size and shape of the piece will depend to some extent on individual preference and on the material examined. The writer generally uses a piece about 5×2–3 mm., which may include the whole of a small lesion or an area cut radially from a large one to include a little healthy tissue at one end. If fruit bodies such as pycnidia can be seen upon the leaf, a strip is cut to include as many as possible. The piece is trans-

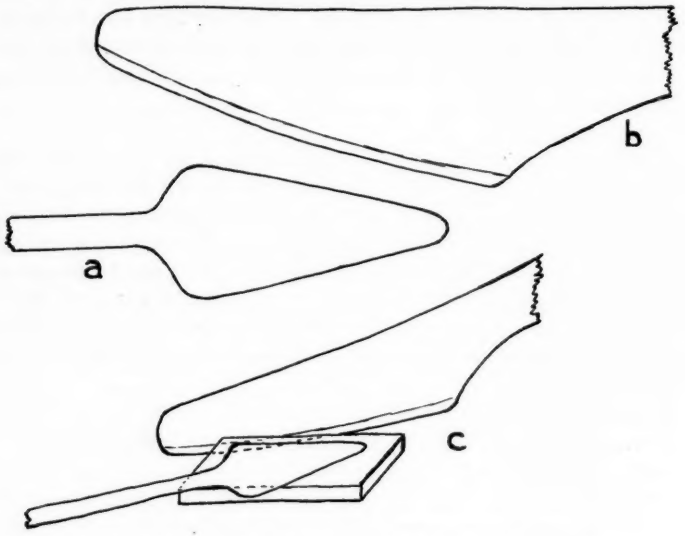

FIG. 1. a. Spear-point needle used to hold piece of leaf on slide.
b. Diagonal scalpel ground with slightly curved blade.
c. Positions of spear-point and diagonal scalpel during cutting of sections.

ferred to a drop of lactophenol at the end of a slide. The glass stage of the dissecting microscope is wiped clean, a drop of water or alcohol is placed in the center of it and the slide is pressed down upon it; this device holds the slide more firmly than do slide clips and leaves the whole surface clear for manipulation. The piece of leaf is drawn from the lactophenol drop toward the center of the slide, where it is briefly moved about to leave it in the middle of a thin film of the fluid.

The piece is now held down with a spear-pointed needle shaped somewhat as in Fig. 1a; this needle need not be sharp, but it should

be smooth and the edges of the blade should be approximately straight in order that one of them may be laid close to that side of the piece from which sections are to be cut. A diagonal scalpel is ground to a slightly curved edge as in Fig. 1b; the edge should be kept keen, but it is neither necessary nor desirable to attempt to bring it to the fine, smooth edge sought in a sectioning razor; a bevel of about 20°, finished and periodically renewed on fine abrasive paper, has been found satisfactory. The diagonal scalpel is used, as shown in Fig. 1c, to chop sections from the side of the piece that projects beyond the edge of the spear-point. The point of the blade is placed in contact with the slide beyond the end of the piece and the handle is lowered until a section has been cut off. The curved edge, used

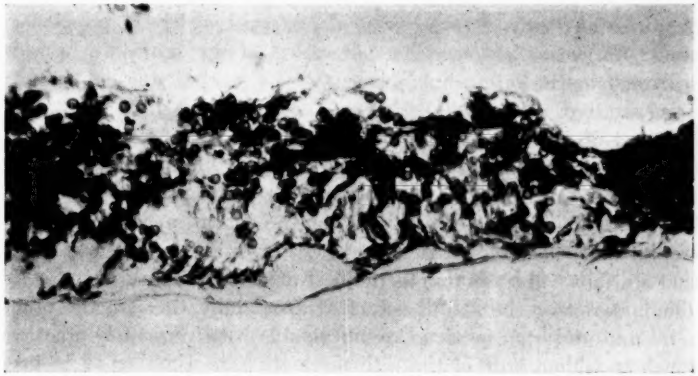

FIG. 2. Freehand section of *Papaver orientale* showing spores of *Entyloma fuscum*, prepared by method described. Dried specimen. Photographed with 16 mm. objective. ×130.

thus, cuts with a shearing action that improves the results; it also allows control of the scalpel during the cut, correction being easily made if the section is seen to be getting thicker or thinner. As each section is cut it is pushed away from the operator toward the far edge of the slide. The whole operation is carried out under the dissecting microscope. If a fairly high magnification is used (60 diameters has been found satisfactory) it is often possible to tell, as each section is made, whether it is useful; thus barren, thick or otherwise useless sections can be removed as made, and only usable sections are left on the slide. When there is some doubt as to whether the fungus is in good fruit, it is often advisable to cut a few sections, remove a thicker slice, cut more sections, and so on; thus the whole piece is sampled.

A point of some importance is the amount of lactophenol left on the slide; if too much is left both piece and sections tend to float out of position, whereas if the slide is practically dry the sections will be damaged as they are pushed across it. Surplus fluid is readily removed with the diagonal scalpel. The writer always keeps a sheet of hard lintless filter paper close to his right hand, for removing surplus lactophenol and pieces of leaf from the scalpel blade, and for cleaning scalpel and spear-point after completing a mount. Lactophenol can be added, if necessary, from the drop at the end of the slide.

Once sufficient sections have been cut and oriented, more lactophenol is added, a cover glass is applied, and the mount is ready for examination.

Figure 2 shows a section, prepared by this method, of the leaf of the oriental poppy, *Papaver orientale*, parasitized by the poppy leaf smut, *Entyloma fuscum*. The teliospores of the smut can be seen scattered in the intercellular spaces. The section was cut from a dried, unfixed, herbarium specimen.

In a wide variety of plants, attacked by various fungi and bacteria, little difficulty has been experienced in obtaining sections thin enough for critical examination with the oil immersion objective. The method is particularly useful for those firm, and often hypertrophied, lesions that are difficult to cut in pith. No claim is made that sections will be as thin as the best of those obtained from favorable material by the skilful use of a razor and pith. On the other hand uniformly fair sections are obtainable with very little practice, which is seldom true of the pith method. The time saved in preparing the mount, to say nothing of the time saved in sharpening a small scalpel as compared with that required for honing a razor, makes the method valuable in the type of work for which it was devised.

It is possible that slight modification would make this method useful for other purposes. Lactophenol has been used by the writer because it is a satisfactory mounting fluid for fungi and possesses the desirable characteristics of low surface tension, moderate viscosity and low rate of evaporation. Possibly mixtures of glycerol and water might prove adequate for the examination of living material; but the method will probably prove most useful with dried material, the firmness of which is preferable to the softness of many living leaves.

SOME EVIDENCE FOR THE SPECIFICITY OF THE FEULGEN REACTION

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ABSTRACT.—**Carr has attacked the specificity of the Feulgen reaction on three grounds: that the chromosomes are adsorbents capable of regenerating the color of the Schiff reagent; that selectivity for the nucleus depends on destruction of cytoplasm by acid hydrolysis preceding staining; and that the reaction is not blocked by SO_2 water, as he says it should be if staining occurs by a chemical reaction. The first point was tested by staining chromosomes treated with nuclease. They were Feulgen negative, but their protein basis remained intact. The second point was tested by hydrolyzing fixed tissues, washing off solutes, drying, and comparing weight loss with controls. As differences were negligible, the fixed cytoplasm must not have been made soluble by hydrolysis. Carr's third point was not tested experimentally. It is concluded that these objections to specificity of the Feulgen reaction are not valid.**

Since the use of the Schiff reagent as a test for thymonucleic acid was first introduced in 1924 by Feulgen and Rossenbeck[1], an extensive literature has appeared concerning its use. As Stowell[2] has recently reviewed this literature, it will not be necessary to review it here; however, most writers have held that, for practical purposes, the Feulgen reaction is specific, with only a few writers dissenting.

In a recent issue of Nature, Carr[3] has maintained that the Feulgen reaction is quite non-specific, and depends upon simple adsorption rather than upon the chemical reaction to which it is usually attributed. His argument rests upon three points. First, the Feulgen color is produced if alumina or other adsorbent is added to the Schiff reagent. As chromosomes ordinarily concentrate stains out of very dilute solutions, they may be regarded as good adsorbents. Hence it is likely that the Feulgen reaction is simply an adsorption. Second, biochemists ordinarily isolate nuclei from cytoplasm by hydrolysis with dilute acids. It therefore seems probable that the selectivity

[1]Feulgen, R., and Rossenbeck, H. 1924. Mikroskopisch-chemischer Nachweis einer Nucleinsäure usw. Zts. physiol. Chem., **135**, 203.

[2]Stowell, R. E. 1945. Feulgen reaction for thymonucleic acid. Stain Techn., **20**, 45.

[3]Carr, J. G. 1945. Mechanism of the Feulgen reaction. Nature, **156**, 143.

of the Feulgen reagent for the nucleus depends upon the destruction of much of the cytoplasm by the acid hydrolysis which precedes treatment with the Schiff reagent. Third, Carr says that, if a chemical reaction between the decolorized fuchsin and an aldehyde were responsible for the reaction, then it ought to be possible to block the reaction by pretreatment of the tissue with SO_2 water. He tried this, using a great excess of SO_2, but the Feulgen reaction was given as usual. Each of these points will be separately discussed.

With regard to Carr's first point, it cannot be denied that the chromosomes are efficient adsorbents, but Carr himself admits that this does not exclude the possibility of a chemical reaction as the basis of staining. The question is, which of two possibilities actually does occur? Experiments of Mazia and Jaeger[4] on salivary gland chromosomes of *Drosophila* show that, if the tissue is first digested with nuclease to remove the thymonucleic acid, the chromosomes become Feulgen negative. Control slides, treated with boiled nuclease, give a typical Feulgen reaction. Staining the digested chromosomes with ninhydrin reagent shows that the protein skeleton of the chromosomes remains intact. Kodani[5] has extended this work to the lampbrush filaments which he has been able to demonstrate within the bands of the salivary gland chromosomes. Each "hair" has a nucleic acid component which can be removed by nuclease, and a protein skeleton which remains intact when the chromosomes are digested with nuclease. The digested hair is Feulgen negative, while the hairs of control slides remain positive. I have verified this work for the lampbrush chromosomes of amphibian eggs. Here again, nuclease action renders the chromosome Feulgen negative without disrupting the protein basis of the chromosomes. It would seem then, regardless of the mechanism of Feulgen staining, that a positive reaction does not occur in the absence of thymonucleic acid.

Carr's second point was also tested experimentally. Rat liver was fixed in Bouin's fluid, washed in 70% alcohol and hydrated, then ground up, and centrifuged to remove excess water. The mash was then divided into three samples, each of which was weighed. Sample A was then dried to constant weight. Sample B was hydrolyzed in 1 *N* HCl at 60°C. for 20 minutes, while sample C was treated with distilled water at the same temperature and for the same length of

[4]van Herwerden, M. A. 1913. Über die Nucleasewirkung aud tierische Zellen. Arch. Zellforsch., 10, 431.

[5]Mazia, D., and Jaeger, L. 1939. Nuclease action, protease action, and histochemical tests on salivary chromosomes of Drosophila. Proc. Nat. Acad. Sci., 25, 456.

time. Samples B and C were washed repeatedly to remove any soluble constituents, then dried to constant weight. Results are tabulated below:

Sample	Wet weight	Dry weight	Percent loss
A	3.914 g.	0.918 g.	76.5
B	3.742	0.849	77.3
C	5.622	1.053	81.3

As the differences in percentage loss are insignificant, it must be concluded that the efficiency of acid hydrolysis in destroying fresh cytoplasm does not extend to fixed tissue, and so selectivity of Feulgen staining cannot depend upon destruction of the cytoplasm. It may be objected that Bouin fixation should not be used in an experiment to test the effect of acid. The choice was made because Bouin's is commonly used before the Feulgen reaction. Also, it must be pointed out that if any cytolytic effect of Bouin's is sufficient to limit Feulgen staining to the nucleus, then practically all nuclear staining must be accounted for on this basis. It is doubtful whether anyone will care to accept this.

In making his third point, Carr starts from the usual assumption that hydrolysis of thymonucleic acid unmasks an aldehyde group. Treatment of such hydrolyzed thymonucleic acid with SO_2 water should then cause the formation of an aldehyde-bisulfite addition product. However, these compounds are notably unstable[6], particularly in the presence of dilute acid or alkali, so it is to be expected that the aldehyde-bisulfite addition product would be in equilibrium with an aldehyde-Schiff-reagent addition product. Because of the intensity of the stain, one would hardly expect to detect dilution of the stain by the usual visual methods. It ought to be possible, however, to find some reagent which would block the aldehyde group irreversibly, and thus give a decisive answer to this problem.

In conclusion, while our knowledge of the Feulgen reaction and its specificity leaves much to be desired, as yet no serious experimental evidence against its practical usefulness in the identification of thymonucleic acid seems to have been brought forth.

[6]See any textbook of organic chemistry.

CURTIS' SUBSTITUTE FOR VAN GIESON STAIN

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Abstract.—**A triple staining method is described in which nuclear staining is by Weigert's hematoxylin. The cytoplasmic and collagen staining is effected by the Curtis substitute for Van Gieson, in which ponceau S is substituted for acid fuchsin. Nuclear staining is sharper than with Delafield's hematoxylin. The red of the collagen fibers is probably not subject to fading. Unlike Van Gieson, this method gives staining of reticular as well as collagen fibers. The advantages of the method are its simplicity and reliability. The use of this method is made possible by a new source of reliable samples of the ponceau S called for in this method.**

In 1889 Van Gieson described a method for staining cytoplasm and collagen differentially by the use of a mixture of picric acid and acid fuchsin. Most references given to this paper are indirect ones or references to abstracts of it.

Weigert (1904) introduced a new and excellent hematoxylin staining method specially designed to resist the acid differentiation occurring during counterstaining with Van Gieson. Curtis (1905) found that ponceau S could be substituted for the acid fuchsin of Van Gieson. Slides stained by his method are less liable to fade. But in Curtis' method, Delafield's hematoxylin was advised.

In this laboratory we have for some years (Carleton and Leach, 1938) used the Curtis' substitute after Weigert's hematoxylin and have obtained satisfactory results; but recently it has become impossible to get reliable samples of ponceau S. Owing to a confusion in nomenclature, completely different dyes were probably supplied.

At my request, Mr. Nantz of the National Aniline Division (Allied Chem. and Dye Corp.) kindly undertook to prepare a sample. I have tested this and find it as good or better than the samples that we used to employ with satisfaction. It is too early to say whether fading will occur.

The following is the method in use in this laboratory:—

1. Fix by any desired fixative.
2. Take sections down to water using iodine and hypo (sodium thiosulfate) if necessary.
3. Stain with Weigert's hematoxylin — 5 to 10 minutes.

   Solution A. 1% hematoxylin in absolute alcohol.

Solution B. 30% ferric chloride in distilled water. 4 ml.
Conc. hydrochloric acid. 1 ml.
Distilled water 100 ml.

Mix 1 part of A with 1 part of B just before use.

Add 2 parts of distilled water.

4. Without acid differentiation, wash in running tap water 5 minutes.
5. Stain 2 to 4 minutes in Curtis' substitute:—
   2% Ponceau S (National Aniline) C. I. 282 5 ml.
   Saturated aqueous solution of picric acid 95 ml.
   2% Acetic acid. 2 ml.
6. Rinse in 96% alcohol.
7. Dehydrate, clear and mount.

Results:—Chromatin — black.
Cytoplasm — yellow.
Collagen and reticular fibers — red.

The results are similar to those given by Van Gieson but both the red and yellow colors are purer and overstaining with the red is less likely to occur. This may be due to the molecular weight of ponceau S exceeding that of acid fuchsin (von Moellendorf, 1922). This would also be the reason why reticular fibers tend to stain by this method (Jalowy, 1937); this is an advantage unless differentiation of collagen and reticular fibers is desired.

The nuclear staining is good and suitable for photomicrography, although not as sharp as a Heidenhain preparation; it is better than after Delafield's which gives such disappointing results with Van Gieson.

This method has some advantages over the numerous other trichrome methods now in use.

a. Simplicity and reliability. First year medical students have been known to get good results.
b. It works well after all fixatives.
c. It probably does not fade.
d. It shows up masses of fibrous tissue clearly for naked eye and low power observation.
e. The nuclear detail is good and in no way obscured by cytoplasmic staining. It is possible to get sharp photomicrographs of both nuclei and collagen fibers in one preparation.

The disadvantage is that cytoplasmic details are not as clearly revealed as after such stains as Masson or Mallory.

My thanks are due to Dr. H. M. Carleton and Mr. T. A. Marsland for their part in the evolution and testing of this method.

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THE USE OF ENZYMES IN THE PREPARATION OF ROOT-TIP SMEARS

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ABSTRACT.—**A simple root-tip smear method for the study of somatic chromosomes of Allium is described, in which an aqueous solution of colchicine and Pectinol, (an enzyme preparation) is used. The root tips are placed in a 0.2% aqueous colchicine solution for 30 minutes, fixed in propionic-alcohol solution, run through a graded series of alcohols, washed thoroughly, treated with a 1% aqueous solution of Pectinol for 1 to 1½ hours, and allowed to stand in water 3 to 5 days to soften. A thin transverse section through the meristematic portion of the root tip is then smeared on a slide in a drop of propiono-carmine stain in the usual way and sealed.**

The use of enzymes in making smear preparations for the study of chromosomes has attracted considerable interest recently. Emsweller and Stuart (1944) described a technic by which enzymes derived from an extract of fungus mycelium and the commercial enzyme preparation Clarase were used in spreading pollen mother cell chromosomes of *Lilium* spp. Frolova (1944) used enzymes in studying the structure of salivary gland chromosomes. Fabergé (1945) found that a mixture of cytase and other enzymes obtained from the stomach fluid of the Roman or edible snail made an excellent macerating medium for smear preparations of root tips and other similar plant tissues. In the present study the commercial enzyme complex, Pectinol, has been used in an attempt to apply to root tips of *Allium* spp. a method similar to that of Emsweller and Stuart (1944) for pollen mother cells.

Because the root-tip chromosomes of *Allium* are long at metaphase, it is desirable in making counts to contract them by the use of colchicine, as described by O'Mara (1939), Burrell (1939), and Emsweller and Stewart (1944). Colchicine suppresses the formation of the achromatic figure so that more figures are obtained with metaphase chromosomes. This treatment not only shortens the chromosomes but makes the constriction region very conspicuous,

as shown in the accompanying illustration (Fig. 1). After fixation for one hour or longer in propionic acid and absolute alcohol, the root tips are carried through the alcohol series and the enzyme according to the schedule that follows. By this method the middle lamella of the cell wall is softened so that the cells separate when smeared on a slide in a drop of propiono-carmine stain. The condition of the cytoplasm seems to be changed to such an extent by the enzyme treatment that by applying very light pressure to the cover slip the cells are flattened and the chromosomes are spread out and separated from one another. Both the ease with which the root tips may be smeared and the sharpness of the stain after treatment with Pectinol are

FIG. 1.—Division figure from a root tip of the amphidiploid species hybrid *Allium cepa* L. × *A. fistulosum* L., showing 32 chromosomes after treatment with Pectinol. × 810.

considered by the authors to be superior to the results obtained by the use of the HCl method described by Warmke (1935).

Four different grades of Pectinol were supplied by the Rohm and Haas Co., Philadelphia, Pa. These are known as Pectinol A, W, M, and 100-D. The first three are for commercial applications while the 100-D is manufactured for experimental purposes only and is a standardized product. These Pectinols are derived from *Aspergillus*, and while their primary action is on pectins they also contain minute quantities of diastases and proteases. A 1% suspension of Pectinol 100-D, in distilled water, was used in this work. This suspension had a reaction of pH 6, and was satisfactory, even though the optimum pH is between 3 and 4.5. Pectinol 100-D is active at room tempera-

ture but its activity increases with rising temperature to approximately 49° C., above which point the activity decreases. In the procedure outlined Pectinols A, W, and M were as effective as the 100-D sample.

The detailed schedule is as follows:

1. Place excised root tips, about 1 cm. in length, in a 0.2% aqueous solution of colchicine for 30 minutes.
2. Wash in water for 15 minutes.
3. Fix in a solution of one part propionic acid to three parts absolute alcohol for 1 hour or longer.
4. Change to 95% alcohol for 15 minutes.
5. Change to 80% alcohol for 15 minutes. If desired the root tips may be stored indefinitely in the 80% alcohol.
6. For immediate study, run the material through a graded series of alcohols: 70, 50, and 25%, respectively, allowing 15 minutes in each. If a shorter schedule is desired, some of these alcohols could probably be omitted.
7. Wash thoroughly in two to three changes of distilled water during 30 minutes to 2 hours. Unless all traces of alcohol are removed, the Pectinol will be inactivated.
8. Place in a 1% aqueous solution of Pectinol for 1 to 1½ hours.
9. Wash in water and allow to stand at room temperature for 3 to 5 days, or until the root tips seem very soft when handled with forceps.
10. Place a thin freehand section of the treated root tip on a slide in a drop of propiono-carmine stain. Smear with a spear-pointed needle or other suitable instrument, cover, warm slightly over a flame, and press gently on the cover slip. If properly treated the root-tip cells should separate and float out in the stain drop as readily as pollen mother cells from an anther.

In cases where the larger root tips were not sufficiently softened to smear readily after the above treatment, they were placed again in a very dilute aqueous Pectinol solution (0.5%) for 1 to 4 hours. After rinsing in distilled water, they were in excellent condition for smearing.

11. For a temporary mount, seal with gum mastic and paraffin. If kept in a refrigerator the preparations are suitable for study for a week or longer.
12. To make the temporary mounts permanent, carefully scrape away the wax, float off the cover slip in equal parts of absolute alcohol and propionic acid, and mount directly in Euparal or Diaphane. This method of making slides permanent was recom-

mended by Burrell (1939) because it involves the least manipulation.

Pectinol was also tested in making smear preparations of pollen mother cells of triploid and tetraploid *Allium* spp. Anthers were fixed in the absolute alcohol and propionic acid solution and run through the alcohol series as described for root tips. After washing thoroughly in distilled water they were treated in 1% Pectinol for 30 minutes and smeared in a drop of propiono-carmine stain in the usual way and sealed. The results were as satisfactory as those obtained by the use of the Clarase method described by Emsweller and Stuart (1944).

The authors have worked almost entirely with a 1% solution of Pectinol but a recent trial indicates that a very dilute solution (0.1 to 0.05%) over a longer period may be equally effective.

The schedule given in this paper has been tested with *Allium* only, but presumably it may be adapted for use with the root tips and similar tissues of other plants.

The authors wish to express their appreciation to Dr. Marion W. Parker for suggesting the use of Pectinol in these cytological studies and for preparing and testing the enzyme extracts used.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

HAUSER, E. A., and LE-BEAU, D. S. **Microscopic studies of lyogels. Preparation of samples for ultra-illumination by incident light.** *Ind. Eng. Chem.*, **38**, 335–8. 1946.

The Ultropak ultramicroscope is used because it permits circular illumination of the preparation by incident light which does not pass through the lenses of the objective. Samples of rubber are dissolved and the solution is spread on a non-miscible liquid. A fine-mesh copper gauze is coated by being drawn upward through the rubber film. Preparations of capillary active substances like soaps are made by dipping the gauze vertically into the solutions. The gauze is permitted to dry and is placed on a microscope slide. Photomicrographs made by using color films are projected to give high magnification, and a tridimensional effect is apparent. Ultraviolet radiation enables the study of fluorescent materials.—*R. T. Whittenberger.*

VOLD, MARJORIE J., and DOSCHER, T. M. **Hot stage for microscopic observations between room temperature and 350° C.** *Ind. Eng. Chem., Anal. Ed.*, **18**, 154–5. 1946.

Commercially available heating stages generally are not serviceable much above 50° C. The hot stage described is constructed of porous fire brick and is suitable for use with 16 mm. and 8 mm. objectives. Temperature is measured with a thermocouple and temperature rise is controlled by means of a variac and a 12-ohm resistance. Temperature difference between center of hot stage and the outermost area varies only $\pm 0.8°$ C. at 250° C. A modification of the hot stage in which an air-driven fan is incorporated gives a temperature distribution that varies only $\pm 0.3°$ C. at 250° C.—*R. T. Whittenberger.*

PHOTOMICROGRAPHY

LEONARD, DONALD W. **Photomicrography: a short method using negative projection prints.** *J. Lab. & Clin. Med.*, **31**, 373–8. 1946.

Negative projection photomicrographs can be produced with very simple equipment available in most laboratories. The microscope, the slide, and the light source should be arranged so that the image is projected onto the photographic paper, preferably contrast paper with a glossy surface. The print obtained will show a tissue section more nearly as it actually exists as it comes from the knife, than will the customary positive photomicrograph, i. e. a thin slice of blood-free white material outlined against a black back ground of space, its delicate pattern of cells and nuclei sharply visible.—*John T. Myers.*

MICROTECHNIC IN GENERAL

BOURDON, P. **L'utilisation des résines artificielles (polystyrolène), comme milieu de montage, avec ou sans lamelles.** *Bull. d'Histol. Appl.*, **19**, 175–6. 1942.

A previous article described the use of a cellulose varnish for mounting preparations which served both as a mounting medium and as a cover slip; but a commercial product was then used which was of indefinite composition and difficult to obtain. A commercial solution of polysterolene which had a very high molecular weight gave excellent results as a varnish, but was unsatisfactory for use with a cover slip because it lost 4/5 of its mass on drying. However, by partial polymerization of styrolene, a product was obtained which was satis-

factory for both purposes. It is prepared as follows: boil gently 100 ml. styrolene until the liquid becomes of a consistency similar to glycerine; extinguish the flame and add 5 ml. of tricresyl phosphate; stopper the flask and chill rapidly. The liquid obtained is colorless, has a refractive index of approximately 1.53 and a viscosity comparable to that of Canada balsam. When used without a cover slip, this medium gives excellent preservation of color.—*Jean E. Conn.*

BOURDON, P. **Sur l'emploi de la cyclohexanone comme agent déshydratant et éclaircissant en technique histologique.** *Bull. d'Histol. Appl.*, **19**, 55. 1942.

Cyclohexanone can be used satisfactorily for dehydration and clearing instead of the traditional absolute alcohol followed by xylene or toulene. This saturated cyclic ketone has a number of advantages: a high boiling point (140° C.), a low melting point (−40° C.), a density close to that of water, miscible with organic solvents and paraffin, only slightly inflammable, non-toxic, and does not harden the tissues.

The procedure used is as follows: Fix and wash tissues as usual. Partially dehydrate in 95% alcohol for 12 hr. Dehydrate and clear in two successive baths of cyclohexanone, the first for 4 hr., the second for 2 hr. Impregnate in two baths of paraffin for 2 hr. or less each. This is satisfactory for pieces 3 mm. thick; for larger pieces more time would be required for each step. The same series of solutions could be used for the dehydration of sections after staining.—*Jean E. Conn.*

DYES AND THEIR BIOLOGICAL USES

GORDON, J., and WALKER, N. **Features in the Congo red molecule associated with the inactivation of complement.** *J. Path. & Bact.*, **57**, 451–6. 1945.

The inactivation of complement appears to result from its adsorption on the dye. Dyes of quite varied constitution can inhibit complement provided they are of sufficient molecular size.—*S. H. Hutner.*

LILLIE, R. D. **Effects of metals on Giemsa stain solutions in fifty per cent glycerol and methanol mixtures.** *J. Lab. and Clin. Med.*, **31**, 253–6. 1946.

Zinc acts on methylated thionins in glycerol-methanol solutions, but not on thionin, to shift their spectral absorption bands toward the red end of the visual spectrum. The use of tin foil or other foils containing tin, zinc or cadmium as a lining for screw caps for Giemsa stain is thus contraindicated. Copper foils containing appreciable admixtures of zinc or tin also seem undesirable. Paraffined cardboard is the most innocuous and generally satisfactory lining for such caps.—*John T. Myers.*

WOLFROM, M. L., HARRIS, W. D., JOHNSON, G. F., MAHAN, J. E., MOFFETT, S. M., and WILDI, B. **Osage orange pigments. XI. Complete structures of osajin and pomiferin.** *J. Amer. Chem. Soc.*, **68**, 406–18. 1946.

The ethylenic linkage located in the C_5H_8 residue of both osajin and pomiferin is conjugate to position 8 of the isoflavone nucleus. The pigments are identical in all respects save for the position of an additional phenolic group in position 3′ of pomiferin. Both pigments are mordant dyes for wool and silk, but have no effect on cotton. The use of tin and chromium as mordants gives especially bright colors.—*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

McMANUS, J. F. A. **The demonstration of certain fatty substances in paraffin sections.** *J. Path. & Bact.*, **58**, 93–5. 1946.

The following technic permits the clear demonstration of certain lipids—probably phospholipids—in sections made by the conventional paraffin method. Fix 1 to 5 weeks in the following solution: cobalt sulfate or nitrate, 1 g. in 80 ml. distilled water; 10 ml. of 10% $CaCl_2$; and 10 ml. formalin. If $CoSO_4$ is used, a precipitate forms, which is covered with cotton and used to support the tissue. If desired post-chrome 24 to 48 hr. in 3% $K_2Cr_2O_7$. Take blocks of tissue 2 to 3 mm. thick, blot dry, and dehydrate in 3 changes of acetone, ½ hr. each. Alcohol

dehydration is also permissible. Place directly in melted paraffin at 55–60° C., change 2 to 3 times in an hour, then block. Cut sections 4 to 6 μ, fix to slides, pass through xylene and 70% alcohol. Stain 30 min. at room temperature in a saturated solution of Sudan black in 70% alcohol. Rinse in 50% alcohol and mount in glycerol. Lipids are deep blue-black.

Material thus stained with Sudan black does not stain with osmic acid or Sudan IV. Myelin is stained. Mitochondria in kidney are clearly demonstrated.—*S. H. Hutner.*

SCHAIN, P. **Cytoplasmic cilia-like extrusions of macrophages as demonstrated by a modified Wright's stain.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* **9.**), 49. 1945.

In pleural and peritoneal fluids cells of reticuloendothelial origin were observed which showed hair-like appendages, resembling cilia. These cilia-like projections from the cytoplasm of the cells were only demonstrable by staining the smears with a modified Wright's stain, or in supravital preparations of freshly removed living cells stained with neutral red and diazine green. The modification of Wright's staining procedure consists of using a diluting fluid buffered to pH 8 instead of 6.8 and of staining at least 10 min. instead of 3 min. The relation of these cytoplasmic extrusions of the reticuloendothelial cells to the formation of normal and immune globulins in the body is briefly discussed.—*L. Farber.*

TILDEN, I. L., and TANAKA, M. **Fite's fuchsin-formaldehyde method for acid-fast bacilli applied to frozen sections.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* **9**), 95. 1945.

A rapid acid-fast staining technic for frozen sections is described which yields suitably stained slides in a much shorter time. The technic recommended is as follows: Fix tissue 24 hr. in 10% formalin or 1:1 mixture of 10% formalin and 95% ethanol; cut frozen sections 10–15μ thick; affix to slide, let stand to near dryness, cover with 95% ethanol, blot and treat 1 min. with absolute ethanol; drain slide, flood with thin celloidin (0.9% solution of dry celloidin in 1:1 ether and absolute ethanol) wipe off excess celloidin around section; before drying is complete place at least 15 min. in carbol fuchsin at room temperature (0.5 g. Magenta III, 5.0 g. phenol crystals, 10 ml. methanol or ethanol, made up to 100 ml. with distilled water); wash, and place 5 min. in 40% neutral reagent grade formalin (the section turns blue); wash, and treat 5 min. with acid alcohol (1% HCl in 70% ethanol); wash, and treat 2–5 min. with 1% aqueous $KMnO_4$ (section turns brown); wash and treat with 2% aqueous oxalic acid 30 sec. or until just a trace of brown color remains; wash and stain with Harris hematoxylin, 2–5 min.; wash, stain with a modified Van Gieson's mixture (acid fuchsin 0.01 g., picric acid 0.5 g., distilled water to 100 ml.), 3 min.; treat with 95% ethanol, 2 min., blot; add 99% ethanol or isopropanol, 1 min., blot; clear in 2 changes of xylene 5–10 min. each; mount in gum damar.

With this technic, nuclei appear brown, muscle yellow, collagenous connective tissue red, acid-fast bacilli deep ultramarine blue and non-acid-fast organisms are unstained.—*L. Farber.*

WACHTENSTEIN. M. **Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells.** *J. Lab.& Clin. Med.*, **31**, 1–17. 1946.

The technic is a modification of Gomori's method for visualizing alkaline phosphatase activity, as follows:

Air-dry blood or bone marrow films as usual. Fix with a solution containing 0.25 g. celloidin in 50 ml. absolute alcohol and 50 ml. ether. Dip in 95% alcohol. Wash in distilled water and place for 15 hr. at 37°C. in a solution consisting of: 3.2% aqueous sodium glycerophosphate ($+5\ H_2O$), 10 ml.; 10% aqueous sodium diethyl barbiturate, 10 ml.; $M/10\ MgSO_4$, 10 ml.; 2% aqueous $Ca(NO_3)_2$, 5 ml.; distilled water, 50 ml.

Wash in water containing a few drops of the calcium nitrate solution, put in a solution of 2% cobalt nitrate for 5 minutes, wash in tap water, and place in a Coplin jar with water containing a few drops of ammonium sulfide. The tissue phosphatase liberates inorganic phosphate from the substrate, and it is precipitated by the calcium ions in the incubating solution. The inorganic phosphate becomes cobalt phosphate, which then becomes black cobalt sulfide. The

number of pus cells as well as the intensity of the phosphatase staining increases with extent of infection, probably due to increased metabolic activity. This activity was markedly decreased in acute myeloid leukemia.—*John T. Myers.*

WEBER, A. **Recherches sur la fixation des tissus adultes ou embryonnaires en vue de l'imprégnation des éléments nerveux par les sels d'argent.** *Bull. d'Histol. Appl.*, **19**, 129–36. 1942.

The author has developed a method of fixation which makes possible excellent silver impregnation of even the finest nerve fibers. His procedure is to fix with formaldehyde in a slightly acid medium for several days. Then the tissues are washed in a mixture of dioxane and propyl alcohol to complete the extraction of lipids. After a rather rapid washing in distilled water, they are ready for impregnation by Bielschowsky's technic. He has succeeded in demonstrating nerve fibers in a variety of tissues including chicken embryos and the brains of fish and of adult rats.—*Jean E. Conn.*

WHITE, B. M., and CULBERTSON, C. G. **A modified Gram's stain for use in staining large numbers of smears.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* 9), 53. 1945.

A Gram-staining method is described for routine use on large numbers of smears of varying densities which gives better differentiation than other procedures tried. The main difference is the use of potassium mercuric iodide as a mordant. The recommended technic is as follows: Stain at least 1 min. with a 1% aqueous filtered crystal violet solution, adding 5 drops of 5% aqueous $NaHCO_3$ solution to each slide; drain and dry by a hot air stream and treat at least 1 min. with 1% aqueous potassium mercuric iodide solution (made by dissolving two potassium mercuric iodide tablets, obtained from Davis and Geck, Inc., 57 Willoughby St., Brooklyn, N. Y. in 100 ml. of distilled water), or just wash off stain with a 1% aqueous potassium mercuric iodide solution and let stand 1 min. or more with the solution; wash with tap water; decolorize with acetone until no more color remains; wash with tap water; counterstain with 0.1% safranin O or Pappenheim's pyronin-methyl-green; wash with tap water and dry.

It is important that the crystal violet should not be washed off with water before the addition of the potassium mercuric iodide. The authors state that the use of this method has eliminated false findings and that a striking differentiation of Gram positive and Gram negative organisms is obtained.—*L. Farber.*

PLANT MICROTECHNIC

WHITTENBERGER, R. T., and BRICE, B. A. **Rapid estimation of rubber in guayule latex dispersions.** *Ind. Eng. Chem., Anal. Ed.*, **18**, 209–10. 1946.

A microscopic method for the rapid estimation of rubber in crude guayule latices is described. The latex is diluted to give 2 to 5 rubber particles per square of a Petroff-Hausser bacteria counter. For one determination, all rubber particles, irrespective of size, are counted in 36 squares in each of two mounts. The spherical rubber particles can be distinguished readily from the non-rubber particles such as chloroplasts, protoplasmic granules, cell wall fragments, bacteria and silt. The method is standardized by making counts on a number of guayule latices of known rubber content, as determined by chemical analysis. After standardization, the concentration of rubber (in mg. per 100 ml.) in an unknown dispersion (C) is calculated by the equation $C = 1.85\ ND$, where N is the observed average number of particles per square in the diluted dispersion, and D is the dilution factor. It is suggested that the method may be useful in selection, breeding, and recovery work on other rubber-bearing plants, including hevea.—*R. T. Whittenberger.*

MICROÖRGANISMS

CAMERON, GEORGE M., and CASTLES, RUTH. **Detection of tubercle bacilli in sputum by the clorox method and the direct smear.** *J. Lab. & Clin. Med.*, **31**, 361–8. 1946.

The direct smear, clorox, and sodium-hydroxide-alum methods for demonstrating tubercle bacilli in sputum were carefully compared. The clorox method

was somewhat more efficient than the NaOH-alum method, giving twice the number of acid fast organisms per field. Both the clorox and the NaOH-alum methods surpass the direct smear. The clorox method is much simpler, gives more complete digestion, and shows less visable extraneous material on the slide. When using the clorox method, a swab should be moistened in clear blood serum before transferring sediment to slides. This provides a background and fixative when only a few acid fast bacilli are present.—*John T. Myers.*

DIAMOND, L. S. **A new rapid stain technic for intestinal protozoa using tergitol-hematoxylin.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* **9**), 68. 1945.

The use of the alipathatic penetrant tergitol No. 7 (Carbide and Carbon Chemicals Corp.) is recommended as a substitute for heat to decrease the hematoxylin staining time. The recommended procedure is as follows:

Prepare a uniform smear of a feces emulsion in physiological salt solution, and immediately fix 2 min. in Schaudinn's solution, to which 5% glacial acetic acid has been added just before use; treat 1 min. with 50% ethanol; wash 1 min. in water; mordant 5 min. in 4% ferric ammonium sulfate solution; rinse quickly in distilled water; stain 5 min. in Hance's (Science, **77**, 287, 1933) tergitol-hematoxylin solution (to a 5% hematoxylin solution in 95% ethanol diluted to 0.5% with distilled water add a "minute quantity" of sodium bicarbonate to ripen and to every 30–40 ml. of this solution add 1 drop of tergitol No. 7 just before use); rinse in distilled water; destain 3–5 min. in saturated aqueous picric acid; rinse in 2 changes of distilled water; treat with potassium acetate solution (the salt added to distilled water until the pH is above 7.0) until blue; treat 1 min. each with 70% and 95% ethanol, and 2 min with absolute alcohol or carbol-xylene; treat twice for 1 min. each time with xylene; mount in balsam, gum damar or clarite.

The smears should never be allowed to dry. For more permanent or special study preparations it is recommended that after fixation the slides be washed a few minutes in an iodine-alcohol solution to remove all mecuric chloride, and that a more gradual alcohol series be used to insure complete dehydration. The staining procedure gives results comparable to those with Heidenhain's iron-hematoxylin method but in a shorter time.—*L. Farber.*

FELSENFELD, O., and YOUNG, V. M. **An improved method for the examination of intestinal protozoa.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* **9**), 47. 1945.

A hematoxylin staining technic is described for use with suitably fixed smears of fresh fecal samples. The fixing solution, a modified Schaudinn's fluid, has the following composition: 200 ml. saturated aqueous $HgCl_2$; 100 ml. 96% ethanol (before use 7 ml. of glacial acetic acid are added to each 100 ml. of this mixture). Slides are delivered to the laboratory in this solution and may be left in it up to 48 hours. The slides are then treated as follows: Immerse 5–10 min. in 96% ethanol containing enough iodine to give a "rich port wine color;" immerse 5 min. in 70% ethanol (if staining cannot be completed the same day slides may be left in 70% ethanol overnight); stain at least 30 min. in Mallory's phosphotungstic-hematoxylin solution (dissolve 0.1 g. hematoxylin for "histologic staining" in 50 ml. of boiling distilled water; when cool mix with 20 ml. 10% aqueous C. P. phosphotungstic acid and make up to 100 ml. with distilled water; add 10 ml. freshly prepared 0.25% aqueous potassium permanganate); immerse slides 5 min. or until smears turn deep blue in a Coplin jar containing distilled water; treat 5 min. each with 70%, 95% and absolute ethanol and xylene; mount with clarite and cover with cover slip.

The smears should not be allowed to dry during the entire procedure.—*L. Farber.*

FUENTES, CESAR. **A method for differentiating Candida albicans in tissue.** *J. Bact.*, **51**, 245–6. 1946.

The fat drops in *Candida albicans* can be stained with fat-soluble dyes and the parasites thus clearly demonstrated in tissue sections. The following method results in much less distortion of both blastospores and hyphae than the methods using alcoholic solutions of the dyes: Stain the thinnest possible frozen sections 5 min. in 1% fresh, filtered Sudan IV in pure (reagent grade?) lactic acid; wash

in distilled water; place sections in 1% aqueous toluidine blue for 2 min.; wash carefully; mount in Apathy's fluid or in a saturated glucose solution. The method was equally effective with fresh tissues or those preserved in 10% formalin for as long as a year.—*Virgene Kavanagh.*

RITTERHOFF, R. J., and BOWMAN, M. G. **Demonstration of tubercle bacilli by fluorescence microscopy.** *Amer. J. Clin. Path.*, **15**, (*Tech. Sect.* **9**), 39. 1945

A comparative study was made of the demonstration of tubercle bacilli by the Ziehl-Neelsen staining method, the fluorescence microscopy procedure, and by animal inoculation.

The methods employed were as follows: Spencer Lens Company fluorescence microscopy accessories were used. For most of the work a 4 mm. objective and a 10× ocular were used, giving a 450 diameter magnification. Later an 8 mm. objective and a 20× ocular were substituted giving a somewhat lower magnification, 400 diameter, but a greater brilliancy of fluorescing organisms. Smears were fixed to slides with egg albumin, air dried and flamed. Slides were stained 2–5 minutes in 0.1% auramine O in 3% phenol, decolorized twice for 2–5 min. with 0.5% HCl in 70% alcohol. No counterstain was used. The tubercle bacilli appeared brilliant yellow on a nearly black background. The faintly fluorescent egg albumin aided in focusing. The Ziehl-Neelsen staining procedure employed was that of "institution 14" in Pottenger's review of staining procedures (Amer. Rev. Tuberc., **45**, 549, 1942). Sputa, gastric contents, feces, urines and spinal fluids were concentrated by the methods of Hanks, Clark & Feldman, (J. Lab. and Clin. Med., **23**, 736, 1938; **25**, 974, 886, 1940). Pleural and ascitic fluids and exudates were centrifuged without previous treatment. Of 2918 specimens examined, 50.5% were Ziehl–Neelsen positive and 60.6% were fluorescent positive. Of these specimens, for example, 11 showed 2 or more fluorescent positive, and Ziehl–Neelsen negative sputa, and 36 patients had earlier sputa positive by fluorescence only and subsequently positive by both staining methods.

The specificity of the fluorescent method was determined by guinea pig inoculations; 106 animals were inoculated with material from 102 cases that were fluorescent-positive but Ziehl–Neelsen-negative. Nine positive tuberculous animals were found, or 8.4%.

The above results indicate that the fluorescence microscopy technic closely approximates the most sensitive methods for demonstrating tubercle bacilli (H. C. Sweany, Amer. J. Clin. Path., **12**, 458, 1942). The specificity of the method does not equal its sensitivity, as evidenced by the low incidence of positive tuberculous animals resulting from inoculation with material that was fluorescence-positive but was Ziehl–Neelsen-negative. The authors conclude that the above results "would obviate the routine use of the fluorescence method because of failure to correspond with the clinical, roentgenographic or pathologic status of the tuberculous patient, and, more importantly, because of the lack of specificity as observed in this investigation."—*L. Farber.*

WELLS, WILLIAM F., WINSLOW, C.-E. A., and ROBERTSON, ELIZABETH C. **Bacteriological procedures in the evaluation of methods for control of air-borne infection.** *Amer. J. Pub. Health*, **36**, 324–31. 1946.

Nasopharyngeal parasites—specifically hemolytic streptococci of the nose and throat—can be isolated in media containing proteose and tryptose peptones. Blood agar containing these ingredients becomes selective for the organisms upon the addition of 2 ppm. of gentian (crystal?) violet, which inhibits most common staphylococci in air. Organisms enriched in proteose-tryptose broth, containing lactose and brom thymol blue to indicate acid production, and ¼ ppm. of gentian violet to inhibit staphylococci, may be transferred to the selective medium for confirmation. Tubes showing acid in 24 or 48 hours are streaked on the gentian violet blood agar, hemolytic organisms being further confirmed as Gram-positive.—*M. W. Jennison.*